STUDIES ON SEX-DETERMINATION IN AMPHIBIANS.

IV. The Effects of External Factors, Acting Before or During the Time of Fertilization, on the Sex Ratio of *Bufo lentiginosus*.

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At the present time practically all of the investigators who are working on the problem of sex-determination believe that the sex of a zygote is fixed at a very early period, and that it is not dependent upon environmental factors acting during embryonic development. There is considerable diversity of opinion, however, regarding the time when sexual differentiation takes place and the way in which it is brought about.

A number of investigators, among whom may be mentioned Rauber ('00), Beard ('02), Schultze ('03) and Russo ('09) maintain that sex is determined in the ovary, chiefly by nutritive conditions. According to this view mature eggs are either male or female, and the spermatozoan can take no part whatever in determining the sex of the egg it fertilizes.

Other investigators, prominent among whom are Stevens ('05), Wilson ('06) and Morgan ('07, '09, '10), are inclined to the opinion that sex is not determined until the time of fertilization, and that it is the spermatozoan that definitely and unalterably fixes the sex of the individual.

These two views are apparently irreconcilable, and such an array of seemingly indisputable facts has been brought forward in support of each of them that it may be, as Jordan ('09) has recently suggested, that "sex has been attained by several paths, and is now determined in different modes and at different times in the different groups of animals and plants." If this be true, it will be futile to try to find a single factor, or set of factors, that is the fundamental cause of sex in all organisms; and investigations in this field must be confined to an attempt to dis-

cover the conditions that determine sex in the particular form selected for study.

The important experiments of Tower ('10) with the eggs of the potato-beetle, *Leptinotarsa*, and also those of Tennent ('10) with cross-fertilized echinoderm eggs, have shown that very definite alterations in the structure of the developing organism can be produced by changing the external conditions that surround the egg at the time of fertilization. The recent investigations of Shull ('10*a*, '10*b*) and those of Whitney ('10) on the rotifer, *Hydatina senta*, indicate that the chemical composition of the water in which the cultures live determines the appearance of the sexual cycle. But what the substances are that bring about the appearance of the sexual forms, and how they act, has not yet been ascertained.

All of these experiments show that environmental factors can greatly change the course of development even if they act for only a comparatively short period on the unsegmented egg: whether they can influence anything as fundamental as sex, however, remains to be determined. It was for the purpose of testing this point that a series of experiments on the eggs of the toad, *Bufo lentiginosus*, was undertaken two years ago. The first of these experiments showed pretty conclusively that temperature, acting at the time of fertilization is not the dominant factor in the determination of sex in this form (King, '10). The present paper records the results of the second series of experiments along this line. It is hoped that the method of investigation employed will eventually settle the point as to whether sex in *Bufo* is determined at or before the time of fertilization, even if it gives no clue to the factors that are involved in this process.

The Wistar Institute of Anatomy and Biology has provided an equipment for the carrying on of these investigations that is nearly ideal for the purpose. This equipment consists of two iron racks, each of which supports three tiers of twenty galvanized iron trays $9 \times 12 \times 2\frac{1}{2}$ inches: dishes of this size are convenient for handling and each gives ample space for fifty large tadpoles. A series of $\frac{3}{4}$-inch iron pipes, connected with the city water system, are attached to the supporting frame five inches above

each tier of trays; and by means of small stop-cocks each dish is supplied with running water. The depth of the water in the trays is kept at about two inches by means of constant level siphons, the water running from each siphon into a trough that empties into a large exit pipe. By the use of this apparatus large numbers of eggs, subjected to very different influences at the time of fertilization, can be reared under similar external conditions. Marked variations in the results of the several series of experiments must be attributed to the different conditions under which the eggs were fertilized; they cannot be due to differences in the environmental conditions under which the embryos developed.

The apparatus described above was used for all of the experiments made during the spring of 1910. An abundant supply of varous kinds of water plants was always kept in the dishes containing the tadpoles. All of the tadpoles received similar food, which consisted of small pieces of cooked meat or of frog muscle, yolk of egg, cereal and so-called "fish food." The water was allowed to run slowly in each dish for about six hours every day, and was then turned entirely off at night to prevent a possible accident through a change in the water pressure. To prevent any loss of tadpoles through a fouling of the water by food and impurities that could not be carried away through the siphon, the inner end of which was covered with fine netting, each dish was thoroughly cleaned once a week during the first month after the experiment was started, and much oftener when the tadpoles had become larger and the weather warm.

As many as possible of the individuals used in each series of experiments were carried through to metamorphosis and their sex ascertained: the methods used in ascertaining sex being the same as those previously employed (King, '07).

In the various tables given in this paper the figures in the second column refer in every case to the number of individuals alive one week after the experiment began; they do not indicate the number of eggs that were subjected to the unusual conditions at the time of fertilization. In investigations of this kind it is very important, as I am fully aware, that the mortality in the

various series should be known and taken into account in considering the results. With the large numbers of eggs that were necessarily used in these studies it was quite impossible to take the time to make an accurate count of the eggs when the experiments were made. For the eggs of the toad, when taken from the uterus, are so closely packed together in long strings of thick jelly-like substance that they are greatly distorted in shape, and a rather careful examination with a lens would be necessary to enable one to tell the exact number of eggs in any given lot.

It is possible that distinct variations in the sex ratios might be obtained when batches of eggs from different females are fertilized with sperm from different males. A control for each series of experiments was therefore considered necessary, and was obtained by fertilizing eggs from the same female with sperm from the same male in ordinary tap water. The percentage of females in this control lot was taken as the standard by which to judge whether the sex ratio had been altered by the changed environmental conditions to which the particular lot of eggs had been subjected at the time of fertilization.

1. The Influence of Alcohol on the Sex Ratio of *Bufo*.

To subject eggs to the action of solutions that would produce marked alterations in the cell structure would seem to be an easy way of ascertaining whether sex can be influenced by external conditions acting at the time of fertilization. Unfortunately the unfertilized eggs of amphibians, as well as those of the different invertebrates that are extensively used for experimental purposes, are very sensitive to the action of various substances, even when used in very dilute solutions for only short periods of time. It is therefore not possible to put the eggs of *Bufo* in any solution strong enough to greatly modify the cell structure without causing immediate death or inducing changes that prevent normal development.

Experiments made by Morgan and Tsuda ('93) show that the eggs of the frog, and also those of the toad, can develop normally for some time if they are placed in a 2.5 per cent. solution of alcohol, although they are not able to live in a 5 per cent. solution. As eggs and sperm, according to Overton ('02), are equally

permeable to the lower alcohols and are not greatly injured by them, the idea was suggested that it might be possible to fertilize the eggs of *Bufo* in solutions of alcohol that would be strong enough to enter the eggs and, by combining with the fats, bring about an alteration in the cell structure that would later manifest itself in a change in the normal sex ratio. Batches of eggs from the same female were therefore placed with sperm from the same male in alcohol solutions of the following strengths: 10, 5, 2, 1, .5, .25, .13 per cent. In each case the eggs remained in the solution for one half hour and were then transferred into fresh water where they continued their development.

None of the eggs that were subjected to the action of the 10 per cent. and the 5 per cent. alcohol segmented. This result can probably be attributed to the injurious effects of the solutions on the spermatozoa which were thus rendered incapable of fertilizing the eggs. Overton ('02) has shown that a 5 per cent. solution of alcohol anæsthetizes muscle, and presumably a solution of this strength would have the same effect on the spermatozoa of *Bufo;* no investigation of this point was made. At least one half of the eggs that were fertilized in the 2 per cent. and in the 1 per cent. solutions of alcohol segmented normally and continued their development: in the remaining series practically all the eggs were fertilized.

Table I. shows the results obtained in this series of investigations.

TABLE I.

EGGS FERTILIZED IN SOLUTIONS OF ALCOHOL.

Per Cent. Alcohol.	Total No. Individuals.	No. Sex Ascertained.	Males.	Females.	Per Cent. Females.
2	398	134	68	66	49.25
1	326	130	69	61	46.92
.5	260	177	81	96	54.23
.25	244	188	95	93	49.52
.13	311	209	100	109	52.15
Control,	210	134	65	69	51.41

The sex ratios obtained in this series are very uniform, since in no case is there a deviation of even five from the percentage of females found in the control lot. Somewhat lower percentages of females were found among the individuals that had developed

from eggs that were fertilized in the stronger solutions; but the deviations of these percentages from that of the control are much too small to be considered as significant, particularly as the lowest proportion of females occurs in the lot in which the eggs has been fertilized in 1 per cent. alcohol and not in the lot where fertilization of the eggs took place in 2 per cent. alcohol. The relatively high percentage of females (54.23) that is found where the eggs had been fertilized in .5 per cent. alcohol is quite within the limits of normal variations in the sex ratio of *Bufo*. For previous studies in which the sex of nearly 10,000 young toads was ascertained (King, '07, '09, '10) show that in any large lot of individuals there is a slight excess of females, as a rule, the proportion of females varying from 51 to 56 per cent. It is evident from the sex ratios obtained in this series of experiments that alcohol, in the strengths used and for the time it acted, has no influence on the determination of sex in *Bufo*.

The growth of the tadpoles that developed from the eggs that were fertilized in 2 per cent. alcohol was greatly retarded. This was not apparent during the first month after the experiment was made, but became very noticeable later. At the time of metamorphosis the majority of these tadpoles had a body length of only 7–8 mm., although the body length of tadpoles of *Bufo* at this period is normally 11–14 mm. During early development the mortality among these individuals was comparatively slight, being not more than 10 per cent. At the approach of metamorphosis the tadpoles, especially the smaller ones, began dying in great numbers; and during the last week of June over fifty of them died from no apparent cause. In no other series of experiments was the mortality among mature tadpoles anywhere near as great as in this case. Alcohol, therefore, although acting for only a short time at the fertilization period in but a 2 per cent. solution, must have so affected many of the tadpoles that they were rendered incapable of undergoing metamorphosis. All of the tadpoles in this lot were normal as far as could be determined without a microscopic examination of the various organs. None of them showed defects in the central nervous system comparable to those that Stockard ('09, '10) has found can be induced in embryos of fundulus by subjecting the fertilized egg to the action

of 3 per cent. alcohol. As many as possible of the tadpoles that died at the time of metamorphosis were preserved; and sections were made of their gonads in the hope that it would be possible to ascertain the sex of the individuals by this means. In very few cases, however, were the gonads sufficiently developed to make a definite determination of sex possible.

The stunting of the individuals and the mortality at the time of metamorphosis was much less evident where the eggs had been fertilized in 1 per cent. alcohol; while in the other lots in the series the tadpoles were as large and as vigorous as were those in the control. In lots of tadpoles that have developed from normally fertilized eggs under similar external conditions there is always considerable variation in the size of the individuals of the same age. Although, as a rule, most of the tadpoles are of medium size, it not infrequently happens that a few giant individuals may be found that are nearly twice as large as the smallest individuals in the lot. It is, therefore, only when the majority of the individuals show marked deviations from the usual size that one can safely attribute the change to the unusual conditions to which the eggs have been subjected.

2. The Influence of the Spermatozoan on the Determination of Sex in *Bufo*.

In his latest contribution to the subject of sex-determination Hertwig ('07) describes a series of experiments in which the eggs of two different frogs were divided into six lots and fertilized with sperm from six different males. The mortality in several of these lots of eggs was very great, and there was no uniformity in the sex ratios that were obtained. Some cultures gave over 90 per cent. of females; others produced an excess of males. In spite of this lack of uniformity, Hertwig considers that the results indicate that the male has some influence in determining sex in *Rana*, although he is of the opinion that, as a rule, "die Eier zur Zeit der Befruchtung sexuell in so hohem Grad determiniert sind, dass der relativ geringe Einfluss des Samens gar nicht zur Geltung kommen wurde."

Morgan ('08) has suggested that, if there be a dimorphism of the spermatozoa of amphibians that is associated with sex-de-

termination, it is possible that more sperm of one kind are produced in some individuals than in others, and that distinct differences in the sex ratios might be obtained if lots of eggs from the same female are fertilized with sperm from different males. There is the possibility, also, that the spermatozoa of one kind, if two kinds exist, are produced only in one testicle, or perhaps in considerably greater numbers in one testicle than in the other. It seemed advisable, therefore, in investigating the possible influence of the spermatozoan on the determination of sex in *Bufo* to carry out the experiment in the following way: a batch of eggs from the right uterus of a female was divided into four lots of from 300–400 eggs each. Each lot of eggs was then fertilized with sperm from the right or from the left testicle of one of three different males.

The sex ratios obtained in this series of experiments are indicated in Table II. In this table the males from which the spermatozoa were obtained are designated as 1, 2, 3; while the letters R.T. and L.T. refer to the right and to the left testicle respectively.

TABLE II.

EGGS FERTILIZED WITH SPERM FROM DIFFERENT MALES.

Males Used.	Total No. Individuals.	No. Sex Ascertained.	Males.	Females.	Per Cent. Females.
1 (L.T.)	255	126	66	60	47.61
1 (R.T.)	403	224	108	116	51.78
2 (L.T.)	380	301	143	158	52.49
3 (R.T.)	221	124	55	69	55.64

Somewhat different percentages of females were obtained in the various lots, as might be expected in any case. As the series stands in the above table, the greatest difference between the percentages of females in any two lots is but 8.03 per cent. If the percentage of females in all of the individuals that developed from eggs that were fertilized with sperm from male 1 (50.28 per cent.) is substituted for the relatively low percentage (47.61) found in the lot where sperm from only the left testicle had been used for fertilization, the difference between the percentages of females at the two extremes of the series falls to 5.36 per cent., which is only 1.19 per cent. greater than that (4.17) found be-

tween the two lots in which the eggs had been fertilized with sperm from the right and from the left testicle of the same individual (male 1). Variations in the percentages of females as great as those shown in the above table have been obtained in other cases where batches of eggs from the same female were fertilized with sperm from the *same* male (Table I.). The results of this experiment, therefore, give no very definite support to Morgan's contention that the male is probably the sex determining factor in amphibians as it appears to be in some of the lower forms. On the other hand, the experiment proves nothing against this theory. For a comparison of the percentages of females in the lot of individuals produced from eggs that were fertilized with sperm from the left testicle of male 1 with that of the corresponding lot in which sperm from the left testicle of male 2 was used shows a difference of but 4.88 per cent.; in the two instances in which sperm from the right testicle was used to fertilize the eggs, the difference in the percentages of females among the young toads is 3.86 per cent.; where one lot of eggs was fertilized with sperm from the right testicle and another lot with sperm from the left testicle of the same individual, there is later a difference of but 4.17 per cent. in the percentages of females in the two lots. It seems evident, therefore, that both kinds of spermatozoa, if two kinds exist, must be produced in approximately equal numbers in each testicle of every male. On the theory that the male is the sex-determining factor in amphibians one would therefore expect to find practically equal proportions of the sexes in any large lot of individuals, whether the eggs had been fertilized with sperm from the same or from different males.

The view that males are produced by the fertilization of eggs with spermatozoa from the right testicle, while females result if eggs are fertilized with spermatozoa from the left testicle has recently been advocated by Von Seligson ('95*a*, '95*b*). This theory, which is generally credited to Hippocrates (460–377 B.C.), is not adequate to explain the determination of sex in *Bufo* since, as shown in the above table, males and females are produced in approximately equal numbers when lots of eggs from the same female are fertilized with sperm from the right or from the left testicle of the same individual.

Of the many investigators who maintain that sex is already determined in the ovary, only von Seligson and Dawson ('09) have advocated the view that the right ovary produces male eggs exclusively and that eggs which develop into females must be derived from the left ovary. Von Seligson believes that only the spermatozoa from the right testicle are able to fertilize male eggs, and, conversely, that eggs from the left ovary must be fertilized by spermatozoa from the left testicle. This, of course, is selective fertilization for which at present there is but little evidence. Dawson is of the opinion that the spermatozoan takes no part whatever in determining sex. All of the eggs that were used in the experiments described above were taken from the right uterus of the same female. They should, therefore, according to Dawson, have produced a great preponderance of males. On the theory advocated by von Seligson two of the lots should have produced a great excess of males; and, unless we assume a struggle for existence between the sex-determining factors in the egg and those in the sperm with a final dominance of one sex or the other, it is difficult to see how the tadpole in the remaining two lots could have any sex at all.

I have not suggested the possibility that in any of these lots the individuals would be exclusively males according to the views of von Seligson and of Dawson, since it is very probable that the right uterus of the toad, and also that of the frog, contains eggs from the left as well as from the right ovary. The eggs of these amphibians break through the walls of the ovary shortly after the time that the germinal vesicle has disappeared preparatory to the formation of the first polar spindle, and they come to lie freely in the body cavity from whence they pass through the oviducts into the uteri. It is very probable that all of the mature eggs are expelled from both ovaries at the same time; and it is not at all unlikely that some of the eggs originating in the left ovary may pass into the right oviduct and vice versa. Presumably, however, the great majority of eggs from the right ovary pass into the right uterus as, owing to the enormous numbers of eggs that are produced, it does not seem possible that many of the eggs can be moved from one part of the body cavity to the other. The results of this series of experiments, which confirm

those previously obtained when batches of eggs from each uterus of a female were fertilized with sperm from the same male (King, '09), show that if two kinds of eggs are produced in the ovaries of *Bufo* they must be produced in approximately equal numbers in each ovary. Experiments similar to these can therefore give no possible clue to the time or to the manner in which sex in *Bufo* is determined.

Hertwig ('07) found that the different lots of frog tadpoles that developed from eggs of the same female that were fertilized with sperm from different males showed marked differences in their growth energy. A similar phenomenon was observed in the various lots of tadpoles of *Bufo* that belonged to this series of experiments. Eggs which were fertilized with sperm from the left testicle of male 2 produced tadpoles which, as a rule, were larger, more uniform in size, and apparently more vigorous than were the individuals in the other lots. The mortality among these individuals was comparatively low. The great majority of the tadpoles that were produced from eggs that were fertilized with sperm from the right testicle of male 3 were somewhat undersized; and they were backward in developing, as only 39 of them had completed metamorphosis by June 22. The mortality in this lot was considerable. Both lots of tadpoles derived from eggs that were fertilized with sperm from the right and from the left testicle of male 1 developed at about the same rate and were of average size. The mortality in these two lots was about 10 per cent.

Owing to a series of accidents a number of individuals in each of these lots were lost; and therefore the comparatively high death rate, as shown in Table II., can be ascribed in great part to this cause. To a similar cause can also be attributed at least 10 per cent. of the mortality in the various other series of experiments described in the present paper.

3. The Effects of Changing the Water Content of the Egg at the Time of Fertilization on the Sex Ratio of *Bufo lentiginosus*.

I. *Extracting Water from an Egg, or Preventing the Absorption of Water by an Egg, during the Fertilization Period.*

If the sex of an embryo is definitely fixed at the time that the egg is fertilized, it is conceivable that the sex-determining process may be of such a character, and so evenly regulated, that placing the egg under conditions that would cause it to lose water or that would prevent its absorption of water during the fertilization period might turn the balance in favor of one sex or the other. Normally, as shown by the investigations of Backman and Runnström ('09), the osmotic pressure of the ovarian egg of *Rana*, and presumably also that of *Bufo*, drops from .48° to .045° when the egg has been fertilized but is still unsegmented. This lowering of the osmotic pressure must be due in great part to the absorption of water by the egg as Bialaszewicz ('08) has recently maintained, although the change is ascribed by Backman and Runnström chiefly to an alteration in the condition of the colloids that is induced by fertilization. Any cause that would tend to prevent the normal increase in the water content of the egg at the time of fertilization might, therefore, produce changes that would be far reaching in their results.

In attempting to ascertain whether extracting water from the egg, or preventing the egg from absorbing water during the fertilization period, would produce any alteration in the sex ratio, two different methods of investigation were employed. For convenience in description these two sets of experiments will be designated as series A and series B.

Series A.—While making experiments with the eggs of *Bufo* in the spring of 1909, a pair of toads was placed in an empty aquarium and left undisturbed for several hours. During this time the female laid a small batch of eggs which were found to be segmenting when the toads were removed from the aquarium for use. The eggs which had thus been normally fertilized out of water were placed at once in a dish of tap water and allowed to

continue their development. The female that had deposited the eggs was killed and her remaining eggs were used for the temperature experiments summarized in Table II. of a previous paper (King, '10). Many of the eggs "fertilized dry" died during the gastrulation period, and only 44 individuals lived until it was possible to ascertain their sex. Of these 44 individuals 17 were males and 27, or 61.36 per cent. were females. The lot of eggs from the same female that serves as a control for this experiment, since they were fertilized with sperm from the same male, produced 51.39 per cent. of females.

The outcome of this single experiment has little significance, as the number of individuals in which sex was ascertained is too small to give results of value, especially as it is not known how long the eggs had been laid and fertilized before they were found and placed in water. From the condition of the eggs when they were discovered it was evident that they had been laid at least three hours. The possibility of obtaining normal tadpoles from eggs that had been fertilized out of water having been demonstrated by this experiment, a further series of experiments was made this past spring in the following way: Strings of ripe eggs were removed from the uterus of a female and spread out in an empty glass dish. Then, by means of a pipette, a few cubic centimeters of a concentrated solution of spermatozoa were distributed drop by drop over the eggs in as uniform a manner as possible. The dish was then covered with a glass plate and set aside. Four hours later part of the eggs (lot X) were placed in water. An examination of the eggs at his time showed that many of them had segmented in an apparently normal manner and were already in the 4–8 cell stage of development. The remaining eggs (lot Y) were transferred into fresh water at the end of seven hours, when a considerable number of them were in the 32 cell stage. A number of eggs in both lots failed to segment, possibly because the lack of water prevented the free movement of the spermatozoa.

In these experiments the loss of water from the eggs as a result of evaporation was probably very slight, since the thick jelly-like substance surrounding the eggs would serve to protect them during the time that they were kept out of water. The condi-

tions under which fertilization was effected, however, prevented the eggs from absorbing any considerable amount of water during this period. The early development of these eggs was not interfered with by the abnormal conditions under which they had been fertilized, as in both series segmentation was normal and but slightly later than that of the control lot. The mortality among the older embryos was considerable; and somewhat greater in lot Y where the eggs had remained the longer time out of water. The results obtained in this series of experiments are brought together in Table III.

TABLE III.

EGGS FERTILIZED DRY.

	Total No. Individuals.	No. of Sex Ascertained.	Males.	Females.	Per Cent. Females.
Lot X (4 hrs.)	243	115	45	70	60.86
Lot Y (7 hrs.)	280	72	21	51	70.83
Control	201	140	65	75	53.57

In each case, as the above table shows, a percentage of females was obtained that is considerably higher than that in the control; the higher percentage of females being found among the individuals of lot Y which had developed from the eggs that had remained the longer time out of water. The possible bearing of these results will be considered in connection with the results of the experiments in series B.

Series B.—The second method used to ascertain whether lessening the water content of the egg at the time of fertilization would produce any alteration in the sex ratio was suggested to me by Dr. E. G. Conklin, and consisted in subjecting the unfertilized eggs to the action of salt or of sugar solutions. These solutions, being hypertonic, would tend to extract water from the eggs or to check the absorption of water by the eggs. To attempt the fertilization of eggs in these solutions would be futile, since Loeb ('92) has shown that the addition of even 2 per cent. NaCl to sea-water anæsthetizes the spermatozoa of *Arbacia*, and a much weaker solution would probably have a similar effect on the spermatozoa of amphibians.

Several preliminary series of experiments made during the spring of 1909 were complete failures, since either the eggs were

not fertilized at all after being subjected to the action of solutions of different strengths for varying lengths of time, or practically all of the eggs that segmented died during the early stages of development. The unfertilized eggs of *Bufo*, as well as those of *Rana* according to the investigations of Hertwig ('95), are very sensitive to the action of hypertonic solutions. It was necessary, therefore, to make other experiments this past spring in order to ascertain the maximum strength of solutions that could be employed and the length of time they could act without injuring the eggs beyond the possibility of their being fertilized and continuing their normal development. The experiments from which results were finally obtained were made as follows: a batch of ripe eggs was transferred directly from the uterus of a female into a 2.5 per cent. solution of c.p. cane sugar and allowed to remain there for ten minutes. During this time the eggs were kept in constant motion, either by stirring them with a glass rod or by gently shaking the dish. The eggs were then placed in a small amount of tap water containing a quantity of spermatozoa. A second lot of eggs from the same female were subjected to the action of a 2.5 per cent. solution of c.p. sodium chloride for the same length of time and were then fertilized with spermatozoa from the male used in the previous case.

Even with the weak solution that was used and for the short time that it acted many of the eggs that had been put in the sugar solution were killed, or at least rendered incapable of being fertilized; not more than one half of the eggs subjected to the action of the NaCl segmented, and many of these did not go beyond the gastrulation stage. Morgan ('06) has shown that the fertilized eggs of the frog can develop normally in a 6 per cent. solution of cane sugar, but that the maximum strength of NaCl that will permit of normal development is not much above 2 per cent. The more injurious action of the NaCl on the eggs of the frog, as well as on those of the toad, is doubtless due, in great part, to the fact that its osmotic pressure is several times greater than that of sugar.

As the unfertilized eggs of *Bufo* are so quickly injured by solutions of salt and of sugar it is difficult to decide what effects these solutions could have had in the short time the eggs remained

in them. Presumably a slight amount of water was extracted from the eggs by the salt solution; but, as the osmotic pressure of sugar is so low, it is very probable that the chief action of the sugar solution would be to prevent the eggs from absorbing water during the fertilization period.

The mortality among the tadpoles reared from the eggs that had been subjected to the action of the salt solution was much greater than when a sugar solution had been used. In both cases the greatest mortality occurred during the first fortnight after the experiment began. The embryos that lived beyond this time seemed for the most part to have recovered from any injurious effects of the treatment to which the eggs had been subjected before fertilization. In their later development the tadpoles in both lots appeared to be perfectly normal and of average size. None of them showed any specific abnormalities such as those Hertwig ('95), Gurwitsch ('95, '96) and Morgan ('03) produced by subjecting the fertilized eggs of the frog or of the toad to the action of solutions of various salts.

The eggs used in these experiments were taken from the left uterus of the female whose eggs, from the right uterus, were employed in the experiments described in section 2; and they were fertilized with spermatozoa from the male designated as no. 2 in those experiments. The sex ratio in the lot of individuals, belonging to the former series, that developed from eggs that were fertilized with spermatozoa from the same male serve as a control for the present experiments (Table II.).

Table IV. shows the results obtained in this investigation.

TABLE IV.

EGGS TREATED WITH HYPERTONIC SOLUTIONS BEFORE FERTILIZATION.

	Total No. Individuals.	No. Sex Ascertained.	Males.	Females.	Per Cent. Females.
Salt (2.5 per cent.)	264	91	25	66	72.52
Sugar (2.5 per cent.)	463	130	39	91	70.00
Control	380	301	143	158	52.49

Both lots, as the above table shows, gave a percentage of females that is considerably higher than that in the control.

The percentage of females among the individuals that developed from the eggs that were subjected to the action of the salt solution before fertilization is greater than that in the lot where a sugar solution had been used. The difference between the two is only 2.52 per cent., however, so it is evident that the sugar solution had practically as great an effect on the eggs as had the salt solution, in spite of the fact that the latter solution has much the higher osmotic pressure.

Although the methods employed to bring about a change in the water contents of the egg at the time of fertilization were very different in the two sets of experiments just described, there is a very striking uniformity in the results. Both series, comprising five lots of eggs from three different females, show a percentage of females much above the upper limit of the range which is apparently normal for the species and which in three instances is nearly 20 per cent. greater than that in the control. These results suggest, although they by no means prove, that extracting water from the egg at the time of fertilization, or preventing the absorption of water by the egg during this period, in some way affects the sex-determining process and tends to the production of relatively more females.

The manner in which the experiments were made, particularly those in series B, seem to preclude the possibility that the spermatozoa and not the eggs could have been affected by the conditions under which fertilization occurred. The results obtained suggest also that the sex-determining mechanism is in the egg and that it can be influenced by external agencies acting during the fertilization period.

II. *The Effects of the Absorption of Water by the Egg at the Time of Fertilization.*

In connection with the experiments just described it seemed worth while to ascertain whether any alteration in the sex ratio of *Bufo* could be induced by subjecting eggs at the time of fertilization to conditions that would tend to cause them to take up more than the normal amount of water. An increase in the amount of water absorbed could presumably be brought about by fertilizing the eggs in solutions of acid or of alkali, since,

according to Loeb ('06), both these substances cause eggs to absorb water, the quantity of water taken up increasing with the quantity of acid or of alkali used.

Of the many preliminary experiments made in the spring of 1909, only one gave results that could be considered of any value. In this case one lot of eggs was artificially fertilized in a .01 per cent. solution of hydrochloric acid: another lot of eggs from the same female was fertilized with spermatozoa from the same male in a .01 per cent. solution of ammonium hydrate. In each case the eggs remained in the solution for one half hour, and were then transferred into fresh water. The mortality in both lots of eggs was very high, and was probably at least 50 per cent. No record was made of the number of individuals with which the experiment was started. The results obtained are indicated in Table V.

TABLE V.

EGGS FERTILIZED IN A SOLUTION OF ACID OR OF ALKALI.

Solution.	No. Sex Ascertained.	Males.	Females.	Per Cent. Females.
Acid.....	124	72	52	41.93
Alkali.....	126	55	71	54.76
Control	200	94	106	53.00

The percentage of females obtained from the lot of eggs that was fertilized it the acid solution is somewhat low, yet one might attribute it to a chance variation in the sex ratio: the percentage of females obtained in the lot of individuals derived from eggs that were fertilized in the alkaline solution is but slightly greater than that in the control, and therefore calls for no comment. These results afford little evidence that the sex ratio of *Bufo* can be altered by subjecting the eggs to environmental conditions at the time of fertilization that might cause them to absorb a greater amount of water than usual.

Hydrochloric acid is inorganic, and ammonium hydrate is known to have a very injurious action on developing eggs. It was thought, therefore, that possibly different results might be obtained if eggs were fertilized in solutions of other substances. The experiments were repeated on a much larger scale this past year; acetic acid and sodium hydrate being

used in making the solutions. Two strengths of each substance were used; a .01 per cent. solution and a .0025 per cent. solution.

In each case eggs and spermatozoa were placed together in the solution and left for one half hour; the dish containing them being kept in constant motion during this time. As the experiment was carried out, therefore, the solutions might have acted on the eggs alone, on the sperm alone, or on both eggs and sperm. After remaining in the solution the given length of time, the eggs were washed in several changes of fresh water and placed in the dishes in which they were to continue their development. The percentage of eggs that failed to develop was about the same in the lots subjected to the action of the acid solutions as where alkaline solutions had been employed; and was greater, as one would expect, where the stronger solutions had been used. It is not possible to say whether the failure of so many of the eggs to segment is to be attributed to the injurious action of the solutions on the eggs, or whether a large proportion of the spermatozoa were rendered incapable of fertilizing the eggs.

For these investigations the eggs of two different females (*a* and *b*) were used. Female *a* was the one from which the eggs were taken that were used in the experiments summarized in Table III., and the control for the former experiments also serves as control for these. Eggs from female *b* were used for the study of the influence of the spermatozoan on the determination of sex in *Bufo*, and also for the experiments in which eggs were subjected to the action of salt and of sugar solutions before fertilization (Table IV.). In the present case eggs from female *b* were fertilized with spermatozoa from both testicles of the male designated as no. 1 in the former investigation (Table II.). The percentage of females that serves as a control in this case was obtained by taking the percentage of females in the total number of individuals in the former experiments that developed from the eggs that were fertilized with spermatozoa from male 1. Only the stronger solutions were used on the eggs taken from female *b*.

The sex ratios obtained in the various lots of individuals derived from eggs that were fertilized in acid solutions are indicated in Table VI.

TABLE VI.

EGGS FERTILIZED IN SOLUTIONS OF ACETIC ACID.

Eggs from	Strength Solution.	Total No. Individuals.	No. Sex Asc.	Males.	Females.	Per Cent. Females.	Per Cent. Females (Control).
Female *a*	Strong (.01 per cent.)	210	79	46	33	41.77	53.57
	Weak (.0025 per cent.)	145	66	42	24	36.30	
Female *b*	Strong (.01 per cent.)	258	71	42	29	40.84	50.28
		613	216	130	86	39.81	

In all cases the number of individuals in which it was possible to ascertain sex was less than one half of that which the lots contained one week after the experiments were started. In spite of this great mortality, due in some cases to accidents, the results are about the same as that obtained where eggs were fertilized in a solution of hydrochloric acid (Table V.). In this series each lot of individuals gave a percentage of females that is considerably lower than that found in the control, and also much below that which appears to be normal for the species. Curiously enough the smallest percentage of females appears in the lot of individuals reared from the eggs of female *a* which had been fertilized in the weaker solution. This result may mean that a solution of the strength employed in this instance is more favorable to the production of males, but more probably it is merely a chance variation in the sex ratio.

The results of this series of experiments are especially interesting and suggestive when compared with those obtained in other cases where eggs from the same females were fertilized under very dissimilar conditions. Eggs from female *a* were fertilized out of water with sperm from the same male used in fertilizing the eggs in this series. In the one case a great excess of females was produced (Table III.); in the other case the percentage of females falls much below normal. Between the two extremes of the series there is a difference of 24.53 per cent. Eggs from female *b* when subjected to the action of a salt or of a sugar solution before fertilization gave in both instances 70.00 per cent. of females; when other eggs from this female are fertil-

ized in acid solutions the percentage of females falls to 40.84 per cent. Between the extremes of the series there is a difference of 31.68 per cent. These variations in the sex ratios are uniform in the different series: they are apparently beyond the limits of normal variations in the sex ratio of *Bufo*, and they are too large to be justly attributed to mere chance variations in the sex ratios of different lots of individuals.

The percentages of females obtained in the series of experiments in which eggs were fertilized in solutions of sodium hydrate are indicated in Table VII.

TABLE VII.

EGGS FERTILIZED IN SOLUTIONS OF SODIUM HYDRATE.

Eggs from	Strength Solution.	Total No. Individuals.	No. of Sex Asc.	Males.	Females.	Per Cent. Females.	Per Cent. Females (Control).
Female *a*	Strong (.01 per cent.)	175	49	20	29	59.18	53.57
Female *b*	Weak (.0025 per cent.)	252	98	42	56	56.12	
	Strong (.01 per cent.)	215	75	33	42	56.00	50.28
		642	222	95	127	57.11	

In this series the apparent effect produced on the sex ratio by fertilizing eggs in alkaline solutions is the reverse of that which seemingly results when eggs are fertilized in acid solutions, since in each case a percentage of females is found which is somewhat above that in the control lot. The percentages of females obtained show very much less deviation from those of the control than was the case in the acid series; and in no lot is the proportion of females more than 6 per cent. above that of the control. It seems probable, therefore, that these percentages, although somewhat high, have no especial significance, and that they are chance variations in the sex ratio.

The great difference between the results obtained when eggs of *Bufo* are fertilized in acid solutions and those found when eggs are fertilized in solutions of sodium hydrate would seem to indicate that these solutions have no effect whatever on the sex-determining process, since both solutions would presumably affect the eggs in the same way and tend to cause them to absorb

water. The rather unusual sex ratios obtained in the acid series would be explained, on this assumption, as due either to chance or to the action of the solution on the spermatozoa. There is, however, another possible explanation that would serve to bring these results in line with those obtained by subjecting eggs to conditions which tend to lessen the water contents during the fertilization period. It is known that egg membranes are more readily permeable to weak acid than to weak alkaline solutions, and as the jelly-like mass that surrounds the unfertilized eggs of *Bufo* is relatively thick, as is also the zona pellucida that incloses each individual egg, it is possible that in these experiments only the acid solutions were able to penetrate the membranes and act on the eggs. That this interpretation of the results is at least plausible is indicated from the sex ratios obtained in the series of experiments about to be described where fertilizing eggs in acid solutions again leads to a seeming increase in the relative proportion of males, while practically normal sex ratios are found where the eggs were fertilized in solutions of NaOH.

In a former study of the effects of temperature on the determination of sex on *Bufo* (King, '10), the results obtained seemed to indicate the possibility that temperature, acting at the time of fertilization, might indirectly influence the sex-determining process; a high temperature favoring the development of females, a low temperature tending to the production of relatively more males. These experiments showed, however, that the temperature at which the eggs are fertilized cannot be the dominating factor in determining sex in *Bufo*, and that at most its influence would be to facilitate or to hinder certain processes taking place in the egg that are concerned with sex-determination.

In the experiments just described the various lots of eggs were fertilized in solutions that were kept at room temperature (20° C.) during the time that they were allowed to act on the eggs. In another series of experiments a batch of eggs from the female whose eggs were used for the alcohol series of experiments (Table I.) was divided into lots of several hundred eggs each. Each lot of eggs was then fertilized with spermatozoa from the same male in a .0050 per cent. solution of acetic acid or of sodium hydrate that was kept at a temperature of 28°, 20° or 11° C. during the

half hour that the eggs remained in it. It was hoped that by this means more certain evidence of the action of temperature in determining sex in *Bufo* might be obtained, since a high temperature would probably facilitate the penetration of a solution into the egg and a low temperature would tend to retard it. Any change in the sex ratio greater than that shown in the other series of experiments in which eggs were fertilized in acid or in alkaline solutions might therefore be attributed to the effects of the temperature at which the solution acted.

A small proportion only of the eggs that were used in these various experiments segmented; and, as in other experiments in which eggs were subjected to abnormal conditions at the time of fertilization, many of the tadpoles died during the early growth period. The results obtained in the acid series are brought together in Table VIII.; the percentage of females given as a control being the same as that for the alcohol series (Table I.).

TABLE VIII.

EGGS FERTILIZED IN ACID SOLUTIONS AT DIFFERENT TEMPERATURES.

Temp. of Solution.	Total No. Individuals.	No. Sex Asc.	Males.	Females.	Per Cent. Females.	Per Cent. Females (Control).
11° C.	156	35	23	12	34.28	
20° C.	104	69	46	23	33.33	51.41
28° C.	135	73	50	23	31.50	
	395	177	119	58	32.76	

In spite of the comparatively small number of individuals in which sex was ascertained, very uniform sex ratios were found in all the various lots, and in every case the percentage of females falls far below that which is apparently normal for the species. The smallest proportion of females was found in the lot in which the eggs had been fertilized at the highest temperature; and, conversely, the greatest number of females appears where fertilization was effected at a temperature of 11° C. This is the result one would expect if the higher temperature had facilitated the penetration into the egg of a solution that could influence the sex-determining mechanism in such a way as to increase its tendency to produce a male. The difference between the percentages of females in the two lots in which the eggs were fertil-

ized at the extreme temperatures is only 2.78 per cent., however, so it is evident that the temperature at which the solution acted had very little, if any, influence on the results.

No evidence regarding the influence of temperature on the determination of sex in *Bufo* is given by the results of these experiments, and their chief interest lies in the fact that they accord so well with the results of the other investigations in which eggs were fertilized in acid solutions. If we consider the series as a whole and disregard the possible influence of temperature on the results, it is found that in the total of 177 individuals in which sex was ascertained only 58, or 32.76 per cent. are females. This is 18.65 per cent. lower than the percentage of females in the control lot and 7.05 per cent. less than the percentage of females obtained in the former series of experiments where eggs were fertilized in acid solutions at room temperature (Table VI.).

The results of the experiments in which eggs were fertilized in solutions of NaOH that were kept at different temperatures during the period that the solutions acted are shown in Table IX.

TABLE IX.

EGGS FERTILIZED IN ALKALINE SOLUTIONS AT DIFFERENT TEMPERATURES.

Temp. of Solution.	Total No. Individuals.	No. Sex Asc.	Males.	Females.	Per Cent. Females.	Per Cent. Females (Control).
11° C.	144	54	27	27	50.00	
20° C.	150	66	28	38	57.57	51.41
28° C.	255	126	65	61	48.41	
	549	246	120	126	51.21	

In this series also the sex ratios obtained indicate that the temperature at which the solutions acted has no appreciable influence on the results, although here, as in the acid series, a slightly lower percentage of females was obtained where the eggs had been fertilized at the highest temperature.

Both lots of eggs that were fertilized at the extremes of temperature used gave a percentage of females slightly below that of the control and some 8 per cent. lower than that in the lot where the eggs had been fertilized at room temperature. In the latter case the relatively high percentage of females cannot be considered as significant, as it is balanced by the low percentages

of females obtained in the other two lots. In the total of 246 individuals in this series in which sex was ascertained 126 were females. The proportion of females in this series is therefore 51.21 per cent., which is remarkably close to that in the control lot (51.41 per cent.).

In none of these experiments does the observed percentage of females differ in any great degree from that of the control. The results therefore agree very well with those of the other series in which eggs were fertilized in solutions of NaOH (Table VII.). Since the fertilization of eggs in solutions of NaOH produce no apparent effect on the sex ratio of *Bufo*, it is evident that either the solutions used were not strong enough to penetrate the egg membranes and act on the egg in the short time in which the eggs remained in them, or that NaOH is not a substance that can act on the egg in such a way as to increase its tendency to produce a male. A further series of experiments will be made in which the eggs are subjected to stronger solutions of NaOH before fertilization. It is probable that the eggs are less readily injured than the spermatozoa by various solutions, and it may be possible to subject them to the action of a comparatively strong solution without serious injury.

Discussion.

The possibility that the relative amount of water in the amphibian egg at the time of fertilization may have some influence in determining sex seems to me to afford a plausible explanation of the unusual sex ratios obtained by Hertwig ('06, '07), and by Kuschakewitsch ('10), in lots of frogs developed from eggs that were overripe when fertilized. In several of his experiments Hertwig obtained from 70–90 per cent. of males; while in one experiment made by Kuschakewitsch, in which the fertilization of a batch of eggs of *Rana esculenta* was delayed for 89 hours, all of the resulting individuals were males. As the mortality in this last lot was only from 4–6 per cent. it is not possible to explain the results as due to selective mortality. Hertwig attributes the great excess of males in these experiments to the "Kernplasmarelation" existing in the eggs at the time that they were fertilized. He believes that young eggs, and also those that

are overripe when fertilized, tend to produce males because they are relatively richer in nuclear substance; females, on the other hand, are produced when the egg is relatively poor in nuclear substance at the time that it is fertilized.

It is very probable that the eggs of the frog normally absorb a slight amount of water during their passage down the oviducts and while they were retained in the uteri. If the eggs remain in the uteri two or three days beyond the time that they would normally have been deposited and fertilized it is not improbable that they continue to absorb water during this time. Overripe eggs, therefore, would contain relatively more water than those that were laid at the normal time. The sex ratios found when such eggs are fertilized accord very well with those that I have obtained by fertilizing the eggs of *Bufo* in acid solutions. In both cases it seems probable that the water contents of the eggs at the time they were fertilized was higher than usual; and one is strongly inclined to attribute the great excess of males to this cause.

Experiments made two years ago with the eggs of *Bufo* to ascertain whether delaying fertilization would produce an alteration in the sex ratio (King, '09) gave negative results. The eggs of *Bufo* are not as favorable as are those of *Rana* for experiments of this kind, as it is not possible to delay the deposition of the eggs more than a few hours. In the experiments referred to a female was killed as she was about to deposit her eggs. The body was not opened until seven hours later when the eggs were artificially fertilized. Post-mortem changes had evidently begun as, although the majority of the eggs segmented in a more or less regular manner, many of them failed to develop beyond the gastrulation stage. The sex ratios were practically normal in the lot of individuals in which sex was ascertained. In the light of these recent experiments the negative results that were obtained can perhaps be atributed to the fact that either the eggs were unable to absorb water during the time that they remained in the uteri of the dead female, or that they were not kept a sufficiently long time to enable them to take up enough water to produce any effect on the sex-determining process.

Unfortunately there is one source of error in all of the experi-

ments described in this paper that makes it impossible to draw any positive conclusions from the results obtained, however definite they may appear in some cases. The mortality in the various lots of individuals was very great and only a small percentage of the eggs with which any experiment was started were carried through to metamorphosis and their sex ascertained. The possibility exists, therefore, that the results may be due solely to chance or to selective mortality, and that in no case was the normal sex ratio really changed by subjecting eggs to the influence of various external factors at or before the time of fertilization. There is, however, no evident relation between mortality and sex in tadpoles reared under artificial conditions, as shown by the investigations of Pflüger ('82) and also by my former work. It does not seem probable, therefore, that in certain lots subjected to similar treatment at the time of fertilization more males than females would always die; while in other lots, where the eggs had received different treatment, the mortality would invariably be greater among the females. Practically normal sex ratios were obtained in all of the control experiments, in the alcohol series, and also in that in which lots of eggs from the same female were fertilized with spermatozoa from different males. This uniformity in the results of so many series of experiments makes it even more improbable that mortality was selective in other cases; neither does it seems possible that the unusual sex ratios obtained in some instances could be chance variations.

The results of the various experiments in which eggs were fertilized in solutions of acetic acid are so uniform that they seem to me very suggestive, even if no definite conclusions from them are possible. Altogether a total of seven lots of eggs from four different females were fertilized in acid solutions. In every instance the percentage of females that was obtained was from 10–20 per cent. lower than that which is probably normal for the species. There is apparently something in this series that stands for a tendency to the production of males rather than of females. These results taken in connection with the equally clear-cut results that were obtained in the five experiments in which water was extracted from the egg, or the egg prevented from absorbing water, would seem to indicate that the relative

amount of water in the egg at the time of fertilization has some influence in determining sex; an increase in water content tending to produce a male; a lowering of the water content favoring the development of a female.

It is, of course, possible to explain these results in other ways besides attributing them to chance or to selective mortality. On the assumption that the male is responsible for sex-determination in *Bufo* the results of the acid series of experiments can be attributed to the fact that the solution killed more of the female-producing than of the male-producing spermatozoa; why this should occur is not at all clear. The explanation of the relatively high percentages of females among the individuals derived from eggs that were subjected to the action of a salt or of a sugar solution before fertilization must be ascribed, according to this view, to the fact that the solutions rendered the eggs more resistant to the entrance of one kind of spermatozoa than to the other. This would be, in effect, selective fertilization for which at present there is no evidence, unless we accept the case of the pigeon. According to the investigations of Aristotle, of Fluorens ('64) and of Cuénot ('99) the first of the two eggs laid by the pigeon almost always produces a male. Selective fertilization therefore very probably occurs in the pigeon if the male is responsible for sex in this form.

That the results of any of these experiments are conclusive, I do not for a moment maintain. They seem to me to be unusual enough, however, to warrant the continuation of investigations along these lines. As they stand the results strongly suggest that sex in *Bufo* is determined at or near the time of fertilization, and that external factors acting during this period may influence the sex-determining mechanism in such a way as to cause it to produce one sex or the other. The results also seem to indicate that in *Bufo* sex is determined in the egg, and that it may depend in some way on the relative amount of water in the egg at the time of fertilization. This suggestion, if capable of more general application, does not exclude the possibility that in some cases the spermatozoan may influence sex. It is known that the unfertilized eggs of the bee produce only males, and that fertilized eggs almost invariably develop into females. In this case the

entrance of the spermatozoan changes the sex, although, strictly speaking, it cannot be said to determine sex, since the egg has a sex mechanism and is capable of developing without fertilization into a male. If the eggs of the vertebrates could develop parthenogenetically it is very unlikely that the embryos would be without sex. It seems probable, therefore, that in all eggs there exists a sex mechanism that alone is capable of giving sex to the resulting embryo; but in many forms besides the bee the spermatozoan may be a sex-changing factor if not a sex-determining one.

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